

## ORIGINAL ARTICLE

## Oestrogen receptor transcripts associated with cervical human papillomavirus infection

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**Objective:** Studies have been inconsistent in establishing sex steroids as a risk factor for human papillomavirus (HPV) infections. This study was designed to determine whether cervical oestrogen receptors (as measured by oestrogen receptor transcripts) at the cervix would be associated with cervical HPV infections.

**Methods:** In 175 women, ages 14–44, we tested for the presence of HPV DNA and oestrogen receptors transcripts at the cervix during routine pelvic examinations. All subjects completed a self administered questionnaire regarding sexual and menstrual histories.

**Results:** 40% of the women (n=70) tested positive for HPV at the cervix. Of those women testing positive for HPV, 99% had detectable levels of Oestrogen receptors transcripts (n=69/70). HPV cervical infections were independently associated with presence of Oestrogen receptors transcripts (OR = 39.8, CI=4.4, 361.1) and greater numbers of sexual partners (OR=1.1, CI=1.01, 1.18).

**Conclusion:** Women who expressed higher levels of oestrogen receptors transcripts were significantly more likely to have cervical HPV infection. These results demonstrate that Oestrogen receptors may play an important part in cervical HPV infections.

Human papillomavirus (HPV) is a common genital infection of females, particularly afflicting adolescents and women in their early 20s. Most infections are subclinical and, hence, the exact prevalence is not known. However, when molecular techniques are used to detect the virus, rates have ranged from 11% to 64% for sexually active adolescents and young adults.<sup>1–6</sup> This has become important because of HPV's strong link to anogenital cancer, particularly cervical cancer. Although HPV is strongly linked to cervical cancer and its precursor, cervical dysplasia, it is clear that other risk factors are associated with acquisition and retention of the virus and viral associated oncogenesis.<sup>7–8</sup> More recently, research has focused on factors influencing HPV persistence at the cervix since those women who have persistent viral infection are at greater risk for developing cervical dysplasia.<sup>6–9</sup> Why some women acquire the infection more easily than others or remain infected is not completely understood.

Both clinical and laboratory studies have suggested that sex hormones can be an additional factor in influencing viral infection.<sup>10</sup> Oral contraceptives and pregnancy have been shown to represent risk factors for acquisition of HPV and cervical dysplasia,<sup>8–11–12</sup> while pregnancy has also been associated with persistence of HPV infection.<sup>13–14</sup> At a cellular level, oestrogen has been shown to stimulate the transcription of HPV-16 early genes in SiHa cells<sup>15</sup> and HPV-16 DNA immortalised human cervical cells.<sup>16</sup> Additionally, oestradiol has shown to activate the upstream regulatory region of HPV-18 in transgenic mice.<sup>17</sup> This stimulatory effect and subsequent gene activation of HPV by oestrogen is thought to occur as a result of the oestrogen receptor complex that binds to the steroid response element within the virus by an unknown mechanism. Whether increased transcription of viral genes is responsible for persistence of the virus or HPV infected cells is not clear.

Although the influence of oestrogen or oestrogen receptors on cervical HPV infection and/or persistence is incompletely understood, we undertook this study to determine whether the expression of oestrogen receptors is associated with cervical HPV infections in humans. Oestrogen receptors were measured since the effects of oestrogen are mediated through

their cognate receptors and it is the oestrogen receptor complex that has been associated with increased transcription of HPV genes. We hypothesised that oestrogen receptors (as measured by oestrogen RNA transcripts) at the cervix were associated with the detection of HPV.

## METHODS

## Study population and study design

Between January 1995 and April 1996, sexually active adolescent and adult females undergoing a pelvic examination in an investigator staffed University of Minnesota Clinic were asked to participate in the study. Adolescents were also recruited from two teen clinics, which serve an urban population, while older women were recruited from a university, and private gynaecological clinics that serve both an urban and suburban population. Participants were enrolled on the basis of clinician/researcher availability. The study was approved by the institutional review board at the University of Minnesota. Written informed consent/assent was obtained from the participants in addition to parental approval, if the subject was under 18 years of age and not seeking confidential care for birth control or diagnosis of a sexually transmitted disease. Subjects were excluded if they refused, denied ever having sexual intercourse, or if they were unable to give consent/assent. Data were not collected on subjects who refused to participate, but refusal rates were minimal (less than 1%).

All subjects completed a self administered questionnaire about sociodemographic and menstrual, sexual, and contraceptive histories before examination. The questionnaire had previously been piloted for test-retest reliability for dating of menstrual and sexual events.<sup>1</sup> Each of the participants underwent a complete pelvic examination by a physician who obtained the Pap smear using a wooden Ayers spatula and cytology brush. The cytology brush and a second cervical brush, used to swab the external cervical os and squamocolumnar junction, were used to elicit nucleic acid DNA and total cellular RNA for cervical HPV and oestrogen receptor transcript detection, respectively. All samples of DNA and total cellular RNA extraction were transported from the clinics at

room temperature and on dry ice, respectively, and then frozen at  $-70^{\circ}\text{C}$  until extraction and processing.

### Nucleic acid extraction and processing

DNA was extracted from the cytobrush sample by submersion of the top half of the brush into white blood cell lysis buffer (Puregene, Gentra Systems Inc, Minneapolis, MN, USA). The solution was then treated with DNase free RNase for 15 minutes at  $37^{\circ}\text{C}$ . Cellular debris was removed by the addition of protein precipitation solution along with vigorous vortexing, followed by centrifugation at  $2000\text{ g}$  for 10 minutes. The supernatant was then collected and transferred to a clean 1.5 ml tube, followed by precipitation of high molecular weight DNA with one volume of isopropanol. The resulting DNA was rinsed once in 80 % ethanol, and allowed to air dry. The DNA sample was resolubilised with a small volume of DNA hydration solution and suspended to homogeneity by gentle rocking overnight at  $20^{\circ}\text{C}$ . Quantitative measures of DNA was conducted by dot quantitation, and stock samples were labelled and stored at  $4^{\circ}\text{C}$  for later use.

RNA was obtained from another cytobrush that was submerged in a RNA processing cell lysis buffer (Puregene, Gentra Systems Inc, Minneapolis, MN, USA). Subsequent processing steps were essentially identical to that for DNA, except that dedicated instruments and reagents from the RNA kit were employed. Cellular RNA was reconstituted in RNA hydration solution and stock concentration were measured by methods of fluorescent spectroscopy. Confirmation of the stock measurement was verified by repeat performance of quantitative fluorescent on serial dilutions of the stock samples.

### Oestrogen receptor transcript detection

Total cellular RNA (30 ng) from each sample was spiked with 1 pg of in vitro transcribed RNA from a clone of a size modified oestrogen receptor cDNA (adenocarcinoma cell line, MCF7). This modified oestrogen receptors (68 base pairs shorter than the normal oestrogen receptors transcript) was created to serve as an internal standard (the internal standard helped to accurately quantitate the oestrogen receptors transcripts and served as a reference against which other RNA values were normalised). These RNA samples and a positive control (with the added internal standard), and negative control were subjected to reverse transcription using random hexameric primers and Mo-Lu reverse transcriptase (Promega, Madison, WI, USA) using the manufacturers recommended reaction conditions. Amplification of the resulting cDNA was achieved by the polymerase chain reaction performed using fluorochrome labelled oligonucleotide primers directed against exons 4 and 6 for 22 cycles. The final PCR products resulted in an internal standard (373 bp), the wild type oestrogen receptors transcript (441 bp) for all the oestrogen receptors positive specimens, and the deletion exon 5 oestrogen receptors transcript variant (305 bp) if present. Internal standard amplified consistently in all specimens yielding a peak at 373 bp in the electrophorogram obtained. The delta 5 oestrogen receptors variant was included since previous studies have shown that the delta 5 oestrogen receptors variant encodes for a novel receptor protein, which binds weakly to oestrogen, but independently is active as a gene transactivator.<sup>18</sup>

With each run of the RT-PCR, a positive control (T47D, an oestrogen receptors positive cell line), was used with Genescan analysis for measuring the transcript. Two  $\mu\text{l}$  of the sample RT-PCR products were analysed on an Applied Biosystems 373 DNA sequencer using the GeneScan (software which quantifies DNA fragments by automated fluorescence). The data from each sample, presented as involving the area under the curve (AUC) of the fluorescent signal, were represented as the quotient of the cellular derived oestrogen receptor

**Table 1** Overall distribution of HPV DNA types

HPV type	No (%)
6	8 (11.4)
11	5 (7.1)
16	27 (38.6)
18	8 (11.4)
31	1 (1.4)
33	1 (1.4)
53	1 (1.4)
58	1 (1.4)
66	2 (2.9)
MM (Pap 291) LVX82	1 (1.4)
MM8 (Pap 155)	1 (1.4)
More than 1 type	8 (11.4)
Unknown type	6 (8.6)

transcript signal over the internal standard, thus normalising the quantitative value. This normalised value was in turn compared to a standard curve of serial dilutions of T47D RNA (positive control) analysed with each run of subject samples. A sample was scored positive for oestrogen receptor transcripts if the normalised signal exceeded the signal of a known negative control.

### HPV DNA detection and typing

One hundred nanograms of DNA were subjected to 40 cycles of polymerase chain reaction (PCR) amplification of the LI open reading frame of HPV using three oligonucleotide primer sets; consensus primer sets MY09/MY11, GP5+/6+ and primer sets specific for HPV types 6, 11, 16, and 18 in separate PCR tubes. Ten  $\mu\text{l}$  of the amplified DNA products were electrophoresed through a 6% polyacrylamide gel in order to detect the presence of HPV DNA. During the MY09/11 PCR, human  $\beta$  globin was simultaneously amplified to serve as an internal control. GP5+/6+ and the type specific primers were used to improve sensitivity for detecting HPV. Although  $\beta$  globin gene cannot be amplified during the GP 5+/6+ PCR, all DNA samples showed successful PCR amplification with MY09/11 primer set. Water was used to serve as a negative control. In all the PCR products positive for HPV DNA further testing for HPV typing was carried out by slot blot assay. Five  $\mu\text{l}$  of the PCR product were denatured and immobilised onto nitrocellulose filters and hybridised with radioactively labelled oligonucleotide probes specific for types 6, 11, 16, 18, 31, 33, and 35. For those specimens not typed by slot blot hybridisation, restriction fragment length polymorphism analysis was performed. The PCR product obtained by MY09/11 primer set amplification was employed as a template. All restriction endonuclease reactions were analysed by 6% polyacrylamide gel electrophoresis.

### Analyses

Statistical analyses was performed using SPSS for Windows software. Bivariate analyses were performed using  $\chi^2$  tests for categorical variables and Student's *t* test, Pearson's correlation, and one way analysis of variance for continuous variables. The variable, oestrogen receptor, was treated as both a dichotomous variable and a continuous variable. Other major independent variables included current age, age of first sex, age at menarche, and number of sexual partners. Both variables, current smoker and pregnancy histories, were treated as dichotomous variables. Use of hormonal contraception and condoms were treated as categorical variables for both lifetime and recent use in the last 6 months (never, one fourth of the time, one half of the time, three fourths of the time, and always). Variables significantly related to HPV and those that approached significance by bivariate analysis or

**Table 2** Comparison of demographic and behavioural characteristics of HPV uninfected (HPV-) and HPV infected (HPV+) subjects

	HPV- (n=105)	HPV+ (n=70)	p Value
Age (years)	24.2	20.4	0.001
Mean age of first sex (years)	17.1	15.5	0.001
Mean age at menarche (years)	12.6	12.3	0.20
Interval between menarche and first intercourse (months)	55.1	39.13	0.02
Number of partners, last 6 months of sexual activity	1.4	1.6	0.29
Number of partners, lifetime	5.3	7.0	0.06
Current smoker: No (%)	36 (36.7)	27 (39.1)	0.75
Ever smoked	58 (59.2)	42 (60.9)	0.83
Ever pregnant: No (%)	42 (40)	19 (27.1)	0.08
Oestrogen receptor status positive	68 (64.8)	69 (98.6)	0.000
Condom use, last 6 months: No (%)			0.32
Never	35 (33.7)	14 (20.0)	
One fourth of the time	15 (14.4)	11 (15.7)	
One half of the time	12 (11.5)	7 (10.0)	
Three fourths of the time	11 (10.06)	9 (12.9)	
All of the time	31 (29.8)	29 (40.2)	
Condom use, lifetime: No (%)			0.03
Never	8 (7.7)	4 (5.7)	
One fourth of the time	30 (28.8)	10 (14.3)	
One half of the time	24 (23.1)	14 (20.0)	
Three fourths of the time	20 (19.2)	12 (17.1)	
All of the time	22 (21.2)	30 (42.9)	

that have been previously shown to be related to HPV, were entered into a multiple logistic regression model to assess their relation to HPV controlling of the influence of other variables. The Wald  $\chi^2$  statistic was used to assess significance for the variables in the multiple logistic regression model and data were expressed in odds ratio (OR) with 95% confidence intervals (CI). Statistical significance was accepted at  $p < 0.05$ .

## RESULTS

### Study population and HPV

A total of 180 women were recruited from the study clinics. Four subjects were dropped from analysis owing to incomplete or invalid questionnaires and one to loss of the HPV specimen, leaving a total of 175 adult women and adolescents available for study analysis. Study participants ranged from 14 years to 44 years of age; 117 were less than 25 years old and 58 were 25 years of age or older. Forty per cent ( $n=70$ ) were positive for HPV DNA at the cervix. Distribution of HPV types using the consensus of both the techniques is shown in table 1. As a multiple or a single infection, HPV 16 was clearly the most prevalent type among the 70 specimens positive for HPV (38.6%). Six of these women had dual infections and two were found to be positive for three types of HPV.

Table 2 compares women with HPV ( $n=70$ ) and without HPV ( $n=105$ ). Women with HPV tended to be younger ( $t = 3.40$ ,  $p=0.001$ ), initiated intercourse at an earlier age ( $t = 3.45$ ,  $p=0.001$ ), had shorter intervals between their menarche and sexual debut ( $t = 2.4$ ,  $p = 0.02$ ), and more lifetime partners ( $t = -1.90$ ,  $p = 0.06$ ). No differences were found among the two groups with respect to use of condoms (recent and lifetime use), oral contraceptives, pregnancy, or smoking history. Of the 148 women who had concomitant Pap smear screening at the time of HPV screening, 10 women had abnormal readings based on the Bethesda System (seven with atypical squamous cells of undetermined significance and three with low grade squamous intraepithelial lesions). Of the seven women with ASCUS, six were HPV positive and all three women with LGSIL were positive for HPV ( $\chi^2 = 13.40$ ,  $p=0.004$ ).

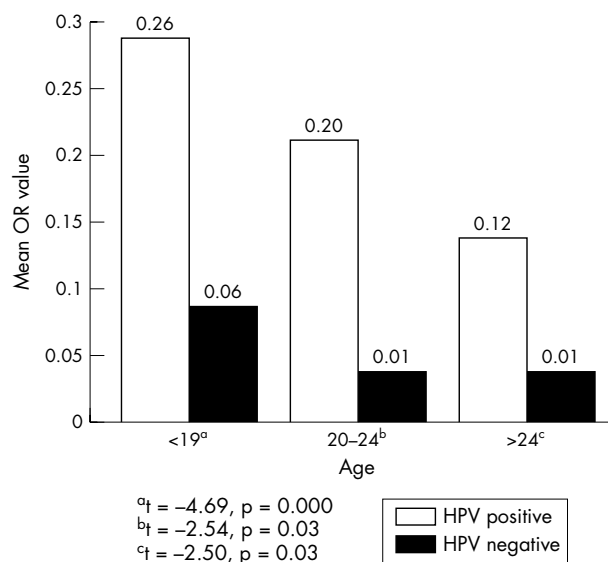
### Oestrogen receptors and its association with HPV

Cervical oestrogen receptor transcripts were detected in 78.3% of the women and, presence of the receptor was inversely cor-

related with age ( $r = -0.26$ ,  $p<0.001$ ). No relation was found between the presence of oestrogen receptors and reported last menses for those women who had a period within the last 30 days. Women who were currently using oral contraceptives ( $n=43$ ) were less likely to be oestrogen receptors positive (62.8%) than women who were not using oral contraceptive (82.2%;  $\chi^2 = 5.97$ ,  $p=0.02$ ). Owing to small numbers, no relations between the presence of oestrogen receptors transcripts and medroxyprogesterone or conjugated oestrogen use could be assessed.

Of those women positive for HPV, 99% had detectable levels of oestrogen receptors transcripts (69/70) while only 64.8% (68/105) of the HPV negative women were positive for oestrogen receptors transcripts ( $\chi^2 = 28.2$ ,  $p<0.001$ ). Given that oestrogen receptors transcripts were measured quantitatively, attempts to examine the relation between expression of oestrogen receptors and HPV acquisition were made (fig 1). Although age was inversely related to being oestrogen receptors positive, women who were HPV positive clearly had higher mean levels of oestrogen receptor transcripts expression (0.223 (SD 0.256)) than those who were HPV negative (0.032 (0.068)) ( $F=52.7$ , 1 df,  $p<0.001$ ).

Multiple logistic regression analysis was used to assess the independent contribution of age, sexual debut, the interval between menarche and sexual debut, number of sexual partners, smoking, and testing positive for oestrogen receptors transcripts to the acquisition of HPV (table 2). When biological, behavioural, and demographic variables were entered simultaneously, the presence of oestrogen receptors transcripts was highly associated with the presence of HPV at the cervix (OR = 39.8, CI = 4.4, 361.1). Greater number of sexual partners also retained its association with HPV (OR = 1.1, CI = 1.01, 1.18) in this model. When oestrogen receptors was entered into the same model as a continuous variable, as opposed to a dichotomous variable, a significant gradient response was found. For each 10% increase in the amount of oestrogen receptors detected there was a three times more likelihood (OR = 3.03, CI=1.84, 4.89) of being infected with HPV. By contrast, earlier sexual debut or shorter intervals between menarche and first sex were not found to be significantly associated to HPV when controlling for the other factors as measured by the models.



**Figure 1** Mean quantitative PCR from oestrogen receptor transcripts correlated to HPV status and age.

**Table 3** Multivariate predictors of HPV infection

Variable	Odds ratio (95% CI)
Current age	0.98 (0.91 to 1.05)
Oestrogen receptor	39.84 (4.40 to 361.05)
Current smoker	0.50 (0.27 to 1.09)
Age at first sex	0.83 (0.62 to 1.12)
Interval between menarche and first sex	1.00 (0.98 to 1.03)
Number of sexual partners	1.10 (1.01 to 1.18)

Note: Odds ratio and CI (confidence intervals) were derived from a final model using the above variables in multivariate logistic analyses.

## DISCUSSION

We found that women who were positive for cervical oestrogen receptors transcripts were significantly more likely to have cervical HPV infection. In reviewing oestrogen receptor physiology, the greatest concentrations of oestrogen receptors has been found in the region of the cervix containing columnar epithelium, with less activity noted in the squamous epithelium.<sup>19-22</sup> Within the squamous epithelium oestrogen receptors seems to have its strongest concentration in the basal layers and diminishes in the upper layers. Oestrogen is clearly higher in perimenarchal females and diminishes with time, but the exact role of circulating oestrogen on oestrogen receptors is unknown. It is clear that activation of oestrogen receptors occurs by the binding of its cognate ligand, E2. In the endometrium, oestrogen receptors is more abundant during times of higher oestrogen influence such as the mid-late proliferative phase of the menstrual cycle.<sup>23</sup> Although a few have reported less oestrogen receptors in cervical tissue during the secretory phase and in postmenopausal specimens,<sup>21, 22, 24</sup> the relation (with the menstrual cycle) has not been as strongly demonstrated for cervical epithelium. We found no relation between menstrual cycle and oestrogen receptors detection, yet clearly the younger women were much more likely to be oestrogen receptors positive.

The ability to respond to oestrogen in the cervix is somewhat limited when compared to the endometrium, yet during pregnancy and in the process of squamous metaplasia some of the cervical cells seem to be very reactive to oestrogenic stimulation.<sup>22, 25</sup> Although the life cycle of HPV is not completely understood, it is thought that the basal cells of the cervical columnar epithelium are more vulnerable to HPV infection due to its physical accessibility.<sup>26</sup> Cervical cell types

sampled were not evaluated in this study, but it is very likely in a overall fairly young population that sampling at the os and squamocolumnar junction resulted in many samples taken from columnar or metaplastic epithelium, because of the positioning of the squamocolumnar junction out on the ectocervix in younger women. If the oestrogen receptor is, in part, responsible for HPV acquisition, this may explain the vulnerability of adolescents, young adults, and pregnant women to HPV infection. Although expression of oestrogen receptors was more common in samples from younger women, the association held for younger and older women alike. Sexual behaviours are clearly contributory risk factors for HPV, yet concerns have been raised that cervical vulnerability from biological factors are just as important with regard to HPV acquisition and cervical dysplasia.<sup>27</sup> Demonstrating that younger age was associated not only with HPV but also with the presence of oestrogen receptors transcripts may lend further evidence that HPV acquisition is not solely the consequence of sexual behaviours but is related to biological vulnerability as well. It may be that the presence of the receptor allows for cellular acquisition of HPV, increased transcription, and/or continued infectivity of the virus. Conversely, it could be that HPV infected cells have greater amounts of oestrogen receptor RNA compared to non-infected cells. Future studies will be needed to clarify the exact interaction or sequencing events related to HPV and cervical oestrogen receptor transcripts found in this preliminary study.

The presence of oestrogen receptors is well established in cervical epithelium and has been reported as well in cervical dysplasia and cancer. The small numbers of abnormal Pap smears prohibited any interpretation with regard to the relation of oestrogen receptor transcripts to dysplasia. Monsenego *et al* found an association with elevated progesterone receptor levels correlating with HPV-16 and HPV-18 positive cervical lesions and oestrogen receptor levels to HPV-6 and HPV-11.<sup>28</sup> In contrast, others have found oestrogen receptor positive cases associated more with viral types 31/33/35.<sup>29, 30</sup> No association was found in our study between viral types and oestrogen receptor transcripts (data not shown). The findings may differ from our results because of the use of different technologies for determining oestrogen receptor and/or the sampling methods. Previous studies relied on methods of immunohistochemistry to detect these receptor proteins and tissue hybridisation for determining HPV type. The present study employed a highly sensitive technique of reverse transcriptase-PCR to measure oestrogen receptor transcripts and PCR based methods for determining HPV type.

Several studies have tried to look at the direct influence of hormone replacement therapy<sup>31, 32</sup> or increased serum oestradiol levels<sup>33</sup> on HPV acquisition or viral load in women. None has found that increased oestrogen levels were associated with an increase in rate or viral load; however, none of these studies looked specifically at the interaction or correlation with circulating oestrogen levels and oestrogen receptors influencing HPV. So what interactions occur at the cervix between exogenous hormones, endogenous hormones, receptors, and HPV are less clear in human models.

Convenience sampling can lead to biases, especially when recruiting from several clinics that serve different populations. We had no reported measure of SES so different population biases can not be assessed. However, there is reason to believe that the sample was representative of women's pubertal maturation; the average age at menarche is the same as that reported in the United States—12.4 years.<sup>34</sup> The relatively high HPV rate in the study population (40%) is most likely secondary to the fact that the study population adolescent and young adult population women was drawn from clinic providing sexual health care. There is no assurance that a single swab will accurately and consistently sample for oestrogen receptors in each woman. Despite this concern, we established a biological gradient between amounts of oestrogen receptors



transcripts present and the presence of HPV that suggest sampling probably occurred in a consistent matter. Finally, transcript expression protein levels do not always parallel mRNA levels so that the findings in this study should be interpreted with that perspective. To date we are unaware of other using this detection method for oestrogen receptors transcripts at the cervix. The method used hopefully provides greater sensitivity and allows specimen collection directly from the patient.

In conclusion, the association found between HPV infection and the presence of oestrogen receptor transcripts is significant for both younger and older women. The results of this present study may lend some insight and support the concept that oestrogen or oestrogen receptors may influence the acquisition of HPV and/or persistent infectivity, although our relatively small sample size, and the consequently wide confidence intervals, somewhat limits the generalisability of these results and should be confirmed by other investigators. Further questions arise from this study on the natural history of HPV and the role of hormonal or hormone and oestrogen receptors interaction that influence on HPV associated infections, increased transcription, and subsequent influence on persistent infection. Studies will be needed to verify these observations and further determine the relation between the presence of oestrogen receptors and circulating oestrogen levels. Prospectively, women will need to be followed to clarify the potential role of oestrogen receptors on continued infectivity with HPV, possible progression to dysplasia, and possible interventions aimed at impeding oestrogen's influence on HPV.

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## CONTRIBUTORS

MLS was responsible for study design, study implementation/data collection, data analysis, and writing of the manuscript; RMcG was involved with study design, laboratory analysis and writing; NZ contributed to laboratory analysis and review of the manuscript; MW and SA contributed to data collection/enrolment and writing; MI was involved with data analysis and data interpretation.

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